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In Vitro Synergistic Effects of Antimicrobial Combinations on Extensively Drug-Resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii* Isolates

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Background: Extensively drug-resistant (XDR) *Pseudomonas aeruginosa* and *Acinetobacter baumannii* are a threat to hospitalized patients. We evaluated the effects of antimicrobial combinations on XDR *P. aeruginosa* and *A. baumannii* isolates.

Methods: *P. aeruginosa* and *A. baumannii* isolates, which were resistant to all antibiotics except colistin (CL), were collected from eight hospitals in Korea. Genes encoding metallo-β-lactamases (MBLs) and OXA carbapenemases were detected by PCR in eight *P. aeruginosa* and 30 *A. baumannii* isolates. *In vitro* synergy of antimicrobial combinations was tested by using the checkerboard method.

Results: Minimum inhibitory concentrations of β-lactams, aminoglycosides, and fluoroquinolones were very high, while that of CL was low for majority of XDR *P. aeruginosa* and *A. baumannii* isolates. Antimicrobial combinations including Imipenem (IPM)-CL, ceftazidime (CAZ)-CL, and rifampin (RIF)-CL exerted only additive/indifferent effects on majority of XDR *P. aeruginosa* isolates. Proportions of XDR *A. baumannii* isolates that showed synergistic and additive/indifferent inhibition after treatment with antimicrobial combinations used are as follows: IPM-ampicillin-sulbactam (AMS), 17% and 80% isolates, respectively; IPM-rifampin (RIF), 13% and 81% isolates, respectively; IPM-CL, 13% and 87% isolates, respectively; and RIF-COL, 20% and 73% isolates, respectively. Significant proportion (19%) of XDR *P. aeruginosa* isolates produced MBLs, and majority (82%) of *A. baumannii* isolates produced either MBLs or OXA-23.

Conclusions: Our results suggest that combinations of IPM-AMS, IPM-RIF, IPM-CL, and RIF-CL are more useful than individual drugs for treating 13-20% of XDR *A. baumannii* infections.

Key Words: *In vitro* synergy, Combination chemotherapy, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*

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INTRODUCTION

Glucose nonfermentative gram-negative bacilli (GNFB) such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii* are opportunistic pathogens that cause infections mainly in hospitalized patients, especially in patients in intensive care units [1, 2].

Most GNFB are naturally resistant to many antimicrobial agents and also acquire antimicrobial resistance easily. Therefore, GNFB infections are difficult to treat.

Carbapenem is the only available drug for treating GNFB infections in many cases. However, the recent increase of carbapenem resistance in GNFB has become a serious problem worldwide, particularly in Korea [3]. Most carbapenem-resistant *P. aeruginosa* and *A. baumannii* strains identified in Korea are also extensively drug-resistant (XDR) [4]. Few treatment options are currently available for treating infections caused by these notorious pathogens. Colistin (CL) and polymyxin B can be used for treating infections caused by XDR *P. aeruginosa* and *A. baumannii*; however, these drugs are associated with severe side effects, including nephrotoxicity and neurotoxicity [5]. Moreover, identification of appropriate therapeutic concentrations of these drugs in the blood is difficult [6, 7]. Tigecycline is another promising antibiotic [8]. However, it cannot be used for treating *P. aeruginosa* infections due to natural resistance [9]. Furthermore, some researchers have reported the emergence of tigecycline-resistant *A. baumannii* in some countries [10, 11]. One classical treatment method often used for treating infections caused by multi-drug resistant (MDR) pathogens is administration of combinations of several antibiotics [12-15]. Some evidence suggests that these combinations are effective for treating infections caused by XDR GNFB.

In this study, we determined the extent of synergistic effects exerted by antimicrobial combinations on XDR *P. aeruginosa* and *A. baumannii* isolates collected from hospitals in Korea.

METHODS

In all, 77 XDR GNFB isolates (43 *P. aeruginosa* and 34 *A. baumannii* isolates), which were resistant to all tested antibiotics, except CL, were collected from eight university hospitals in Korea in 2007. Antimicrobial susceptibilities of these isolates were initially tested at each hospital by using CLSI disk diffusion method or Vitek 2 system (bioMerieux, Marcy l'Etoile, France).

The species of each isolate was determined at a coordinating laboratory, and minimum inhibitory concentration (MIC) of each antibiotic (piperacillin, piperacillin-tazobactam, ampicillin-sulbactam [AMS], cefotaxime, ceftazidime [CAZ], cefepime, aztreonam, imipenem [IPM], meropenem, gentamicin, amikacin, ciprofloxacin, trimethoprim-sulfamethoxazole, and CL) was determined by using CLSI agar dilution method [16].

The ability of each isolate to produce carbapenemases and metallo-β-lactamases (MBLs) was screened by Hodge test [17] and IPM-EDTA sodium mercaptoacetic acid double-disk synergy test [18], respectively. Results of these tests were confirmed by PCR to determine the presence of bla_{VIM}, bla_{IMP}, bla_{SIM}, and bla_{OXA} (bla_{OXA-23}, bla_{OXA-24}, bla_{OXA-51}, and bla_{OXA-58}) [19]. DNA was extracted from whole-cell lysates by boiling bacterial colonies. Amplification was performed in a 20-μL reaction mixture containing 1-μL heat-extracted DNA template, 10 pmol of each primer, and PreMix (Bioneer, Cheongwon, Korea) containing 1 U Taq DNA polymerase. Sizes of amplified products were confirmed by performing electrophoresis, and each confirmed amplified product was sequenced twice by using an automatic sequencer (model 3730xl; Applied Biosystems, Weiterstadt, Germany).

Checkerboard method was used to determine the degree of *in vitro* synergistic effects exerted by antimicrobial combinations used on 30 randomly selected isolates of XDR *P. aeruginosa* and *A. baumannii* each [20]. Antimicrobial combinations tested were IPM and CL, rifampin (RIF) and CL, and CAZ and CL for *P. aeruginosa* and IPM and CL, IPM and AMS, IPM and RIF, and RIF and CL for *A. baumannii*. Fractional inhibitory concentration index (FICI) was calculated by using the following formulae:

FIC_A = MIC of drug A in combination/MIC of drug A alone
FIC_B = MIC of drug B in combination/MIC of drug B alone
$FICI = FIC_A + FIC_B$

FICI were interpreted as follows: ≤0.5, synergistic effect; 0.5-4, additive/indifferent effect; and ≥4, antagonistic effect. *Escherichia coli* (ATCC 25922) and *P. aeruginosa* (ATCC 27853) strains were used for quality control.

RESULTS

None of the XDR GNFB isolates examined was susceptible to any of the tested antibiotic, except CL. The MICs of β-lactams, aminoglycosides, and fluoroquinolones were very high for majority of XDR *P. aeruginosa* and *A. baumannii* isolates (Table 1). However, 38% (3/8) of MBL-producing *P. aeruginosa* isolates were intermediately sensitive to aztreonam and 86% (6/7) of OXA-23-negative *A. baumannii* isolates were intermediately sensitive to IPM.

Of the 43 XDR *P. aeruginosa* isolates, four were IMP-1- and VIM-2-producing isolates. Of the 34 XDR *A. baumannii* isolates, two were IMP-1-, VIM-2-, and SIM-1-producing isolates. Most MBL-negative XDR *A. baumannii* isolates yielded positive results for bla_{OXA-23} (82%), and all the *A. baumannii* isolates yielded positive results for bla_{OXA-51}.

Table 1. Antimicrobial susceptibilities of extensively drug-resistant *P. aeruginosa* and *A. baumannii* isolates

Antibiotic	*Pseudomonas aeruginosa* (N=43)						*Acinetobacter baumannii* (N=34)					
	MIC (μg/mL)			Susceptibility (%)			MIC (μg/mL)			Susceptibility (%)		
	Range	50%	90%	S	I	R	Range	50%	90%	S	I	R
Piperacillin	128 to >256	>256	>256	0	0	100	>256	>256	>256	0	0	100
Piperacillin-Tazobactam	128 to >256	>256	>256	0	0	100	>128	>128	>128	0	0	100
Ampicillin-Sulbactam	NT						32 to >128	64	>128	0	0	100
Cefotaxime	NT						>128	>128	>128	0	0	100
Ceftazidime	16 to >128	64	>128	0	0	100	128 to >128	128	>128	0	0	100
Cefepime	32 to >128	128	>128	0	0	100	32 to >128	>128	>128	0	0	100
Aztreonam	16 to >128	128	>128	0	19	81	NT					
Imipenem	8 to >128	32	32	0	12	88	8-32	32	32	0	6	94
Meropenem	8 to >128	>128	>128	0	2	98	32-64	32	64	0	0	100
Gentamicin	64 to >128	>128	>128	0	0	100	>128	>128	>128	0	0	100
Amikacin	32 to >128	>128	>128	0	0	100	>128	>128	>128	0	0	100
Ciprofloxacin	8 to >128	32	64	0	0	100	32-128	64	128	0	0	100
Trimethoprim-Sulfamethoxazole	NT						16 to >128	128	>128	0	0	100
Colistin	0.5-1	0.5	1	100	-	0	0.5-1	0.5	1	100	-	0

Abbreviations: MIC, minimum inhibitory concentration; NT, not tested; R, resistant; I, intermediate; S, susceptible.

Table 2. Effects of antimicrobial combinations on extensively drug-resistant *P. aeruginosa* isolates

MBL type	N of tested	N of isolates (%) with synergistic effect on								
		Imipenem-Colistin*			Ceftazidime-Colistin†			Rifampin-Colistin		
		Syn	Ad/In	Ant	Syn	Ad/In	Ant	Syn	Ad/In	Ant
MBL negative	22	0 (0)	22 (100)	0 (0)	0 (0)	22 (100)	0 (0)	0 (0)	22 (100)	0 (0)
MBL positive	8	0 (0)	7 (88)	0 (0)	0 (0)	8 (100)	0 (0)	0 (0)	8 (100)	0 (0)
IMP positive	4	0 (0)	4 (100)	0 (0)	0 (0)	4 (100)	0 (0)	0 (0)	4 (100)	0 (0)
VIM positive	4	0 (0)	3 (75)	0 (0)	0 (0)	4 (100)	0 (0)	0 (0)	4 (100)	0 (0)
Total	30	0 (0)	29 (96)	0 (0)	0 (0)	28 (93)	0 (0)	0 (0)	30 (100)	0 (0)

*FICI value of one strain in the VIM-positive group could not be calculated; †FICI values of two strains in the MBL-negative group could not be calculated.
Abbreviations: FICI, fractional inhibitory concentration index; Syn, synergistic (FICI, ≤0.5); Ad/In, additive/indifferent (FICI, 0.5-4); Ant, antagonistic (FICI, ≥4); MBL, metallo-β-lactamase.

In the synergy test, FICI values for the antimicrobial combinations used could not be calculated for three *P. aeruginosa* isolates and five *A. baumannii* isolates because their MICs exceeded the expected values. Three antimicrobial combinations exerted only additive/indifferent effects on all XDR *P. aeruginosa* isolates irrespective of their MBL production status (Table 2). Proportions of XDR *A. baumannii* isolates that showed synergistic and additive/indifferent inhibition after treatment with the antimicrobial combinations used are as follows: IPM-AMS, 17% and 80% isolates, respectively; IPM-RIF, 13% and 81% isolates, respectively; IPM-CL, 13% and 87% isolates, respectively; and RIF-CL, 20% and 73% isolates, respectively (Table 3). Most *A. baumannii* isolates that showed synergistic inhibition produced MBLs. The extent of decrease of MICs in *A. baumannii* isolates ranged from one quarter to one sixteenths (FICI, 0.25-5). MICs of most antimicrobial concentrations tested decreased from high-level resistance range to susceptible and intermediate range (Table 4). None of the tested combinations exerted antagonistic effects.

DISCUSSION

The recent increase in XDR GNFB infections in health-care settings has threatened public health in many countries. In Korea,

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Table 3. Effects of antimicrobial combinations on extensively drug-resistant *A. baumannii* isolates

Carbapenemase type	N of tested	N of isolates (%) with synergistic effect on											
		Imipenem-Ampicillin-Sulbactam*			Imipenem-Rifampin†			Imipenem-Colistin			Rifampin-Colistin†		
		Syn	Ad/In	Ant	Syn	Ad/In	Ant	Syn	Ad/In	Ant	Syn	Ad/In	Ant
MBL negative	24	1 (4)	22 (92)	0 (0)	1 (4)	23 (96)	0 (0)	2 (8)	22 (92)	0 (0)	3 (13)	21 (87)	0 (0)
OXA-23 positive	16	1 (6)	15 (94)	0 (0)	1 (6)	15 (94)	0 (0)	2 (13)	14 (87)	0 (0)	3 (18)	13 (82)	0 (0)
OXA-23 negative	8	0 (0)	7 (88)	0 (0)	0 (0)	4 (50)	0 (0)	0 (0)	8 (100)	0 (0)	0 (0)	8 (100)	0 (0)
MBL positive	6	4 (67)	2 (33)	0 (0)	3 (50)	1 (17)	0 (0)	2 (33)	4 (67)	0 (0)	3 (50)	1 (17)	0 (0)
IMP positive	2	1 (50)	1 (50)	0 (0)	1 (50)	1 (50)	0 (0)	1 (50)	1 (50)	0 (0)	2 (100)	0 (0)	0 (0)
VIM positive	2	2 (100)	0 (0)	0 (0)	2 (100)	0 (0)	0 (0)	1 (50)	1 (50)	0 (0)	1 (50)	1 (0)	0 (0)
SIM positive	2	1 (50)	1 (50)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (100)	0 (0)	0 (0)	0 (0)	0 (0)
Total	30	5 (17)	24 (80)	0 (0)	4 (13)	24 (81)	0 (0)	4 (13)	26 (87)	0 (0)	6 (20)	22 (73)	0 (0)

*FICI value of one isolate in OXA-23-positive group could not be calculated; †FICI values of two isolates in SIM-positive group could not be calculated.
Abbreviations: FICI, fractional inhibitory concentration index; Syn, synergistic (FICI, ≤0.5); Ad/In, additive/indifferent (FICI, 0.5-4); Ant, antagonistic (FICI, ≥4); MBL, metallo-β-lactamase.

Table 4. Concentrations of antibiotic combinations that exerted synergistic effects on extensively drug-resistant *A. baumannii* isolates

Combination (N of isolates tested)	Strain No.	MICs of individual antimicrobials (μg/mL)		MICs in combination (μg/mL)		Effect of combination
Imipenem-Colistin (4)		Imipenem	Colistin	Imipenem	Colistin	FICI
	9	32	2	4	1	0.375
	11	16	2	4	1	0.5
	25	32	0.5	8	0.12	0.5
	26	64	4	8	0.5	0.25
Imipenem-Ampicillin-Sulbactam (6)		Imipenem	Ampicillin-Sulbactam	Imipenem	Ampicillin-Sulbactam	FICI
	19	32	64	8	16	0.5
	25	32	>128	8	32	0.5
	27	32	8	8	1	0.375
	28	8	4	2	1	0.5
	29	16	4	4	1	0.5
	30	64	8	16	2	0.5
Imipenem-Rifampin (4)		Imipenem	Rifampin	Imipenem	Rifampin	FICI
	12	64	32	16	8	0.5
	25	32	4	8	0.5	0.375
	28	16	8	4	2	0.5
	29	16	4	1	1	0.3125
Rifampin-Colistin (6)		Rifampin	Colistin	Rifampin	Colistin	FICI
	2	4	0.5	1	0.12	0.5
	9	32	2	1	0.5	0.156
	11	32	1	8	0.25	0.5
	25	4	0.5	1	0.12	0.5
	26	64	2	8	0.5	0.375
	29	4	0.5	0.5	0.12	0.375

Abbreviations: MIC, minimum inhibitory concentration; FICI, fractional inhibitory concentration index.

the recent rates of carbapenem resistance are moderate to high for *P. aeruginosa* and *A. baumannii*. Moreover, MDR and XDR *P. aeruginosa* and *A. baumannii* isolates are commonly identified in Korea. Carbapenem resistance can result from enzyme production, porin loss, and active efflux. However, it is difficult to determine the exact mechanisms underlying carbapenem resistance, except enzyme production. Mechanisms underlying carbapenem resistance could not be determined for most *P. aeruginosa* isolates, except eight (19%) MBL-producing isolates (Table 2). Porin loss and active efflux might be the reasons for carbapenem resistance in most *P. aeruginosa* isolates examined in this study. Majority of XDR *A. baumannii* isolates produced β-lactamases, including MBLs and OXA-23 (Table 3).

Polymyxin B and polymyxin E (CL) are regarded as the last resort for treating infections caused by MDR or XDR gram-negative pathogens because of the recent spread of antibiotic resistance in many gram-negative bacilli even though severe toxicity. Two studies have suggested that polymyxin B and CL are good treatment options because many XDR *P. aeruginosa* and *A. baumannii* isolates were susceptible to these drugs [21, 22], which was consistent with the present results (Table 1). Resistance to polymyxins has been rarely observed recently; however, several mechanisms through which bacteria may acquire resistance to polymyxin B and CL have been proposed [23, 24]. A study by Matthaiou *et al*. [25] showed a relationship between inappropriate use of CL and development of resistance in *P. aeruginosa* and *A. baumannii*. Furthermore, Kim *et al*. [26] recently reported that mutations in *pmrB* could induce *in vivo* emergence of CL resistance in *A. baumannii* clinical isolates of sequence type 357. Thus, the threat of increasing resistance to polymyxin B and CL is a problem because these drugs need to undergo susceptibility testing before their use in clinical settings. Moreover, limitation of susceptibility method to these drugs may be problematic in clinical microbiology laboratories [27, 28]. Treatment with polymyxin B and CL has resulted in frequent nephrotoxicity and neurotoxicity, especially in patients with deteriorated renal function. Therefore, close monitoring and caution are often requested during their use. Combination therapy with antibiotics is often used for treating infections caused by MDR or XDR pathogens [12-15]. Because antimicrobial combinations exerting synergistic effects enable the use of reduced concentrations of individual drugs, such combinations may decrease the possible toxicities associated with high drug concentrations. Polymyxins act primarily on the cell wall by inducing rapid changes in the permeability of the cytoplasmic membrane of gram-negative bacilli, thereby permitting the entry of other antimicrobial agents into the cell. Various antimicrobial agents can be used in combination with CL. Antibiotics that are most frequently combined with CL include β-lactam antibiotics such as carbapenem and RIF. Landman *et al*. [29] reported that the combination of polymyxin B with IPM or RIF exerted synergistic effects in 80% and 90% cases, respectively, in a time-kill study on *P. aeruginosa*. In a study by Gunderson *et al*. [30], the combination of CL with CAZ exerted synergistic effects on two CL-susceptible MDR *P. aeruginosa* isolates. However, inconsistent results have been obtained by using the combination of carbapenems with CL for treating infections caused by MDR *P. aeruginosa*. Two studies have found that the combination of CL with meropenem only exerted additive/indifferent effects on MDR *P. aeruginosa* [31, 32]. A recent meta-analysis by Zusman *et al*. [15] involving 39 publications and 15 conference proceedings related to the *in vitro* examination of the combinations of polymyxins with carbapenems showed that the combination of polymyxins with IPM exerted synergistic effects on 60% *P. aeruginosa* isolates tested and antagonistic effects on 21% *P. aeruginosa* isolates tested.

Use of combination therapy can suppress the development of resistance *in vitro*. In this study, both IPM-CL and CAZ-CL only exerted additive/indifferent effects and did not exert synergistic effects on all XDR *P. aeruginosa* isolates irrespective of their MBL production status (Table 3). The combination of CL with RIF is generally recommended in regimens for treating infections caused by MDR and XDR gram-negative pathogens. However, the rates of synergy exerted by RIF-CL against different *P. aeruginosa* isolates ranged from 5.7% (2/35) to 16.6% (1/6) [31, 33]. Moreover, RIF-CL did not exert synergistic effects on any *P. aeruginosa* isolate in the present study (Table 2). The reason for this discrepancy is unknown, and further evaluation may be required to completely determine the effect of RIF-CL on various *P. aeruginosa* isolates.

The combination of sulbactam, which is effective against *A. baumannii* [34], with IPM exerted a synergistic effect on approximately 17% (5/30) XDR *A. baumannii*, of which four isolates produced MBLs. RIF exhibits bactericidal activity against *A. baumannii in vitro*. A study by Timurkaynak *et al*. [31] showed that 64% of 25 MDR *A. baumannii* isolates were susceptible to RIF. Combinations of RIF with β-lactam antibiotics were effective in mouse model of *A. baumannii*-induced pneumonia [35, 36]. However, IPM-RIF only exerted a synergistic effect on as low as 13% (4/30) XDR *A. baumannii* isolates in our study. Many studies have shown that CL monotherapy is effective against MDR and XDR *A. baumannii* and that CL-RIF exerts synergistic and

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bactericidal effects [37, 38]. In this study, the rates of synergy exerted by IPM-RIF and RIF-CL were 13% and 20%, respectively (Table 3).

A study conducted in the UK by Wareham *et al*. [39] reported that combinations of polymyxin B with IPM, RIF, or azithromycin did not exert any synergistic effect on OXA-23-producing MDR *A. baumannii* isolates. We also observed that the rates of synergy of antimicrobial combinations were higher in MBL-producing *A. baumannii* isolates than in MBL-negative *A. baumannii* isolates (Table 3). However, a recent meta-analysis showed that combinations of polymyxins with IPM exerted synergistic effects on 56% *A. baumannii* isolates tested [15]. Among the antimicrobial combinations that exerted synergistic effects, MICs of IPM, AMS, and RIF decreased from resistant or intermediate range to susceptible range (Table 4). Moreover, the MIC of CL when used in combination decreased by 2-8 times compared with that when used alone. This finding implies that infections caused by isolates that are susceptible to the synergistic effects of antimicrobial combination can be treated by using conventional treatment regimens even when these isolates are resistant to individual drugs. This also means that the possibility of CL toxicity can be reduced. A recent retrospective cohort study involving 236 patients with XDR *A. baumannii*-induced pneumonia found that survival rates (in terms of 28-day mortality) of patients treated with combinations of CL with sulbactam, tigecycline, or carbapenem were superior to those of patients in the control group who were not treated with any active agent against XDR *A. baumannii* [40].

In conclusion, significant proportion of XDR *P. aeruginosa* isolates produced MBLs and majority of *A. baumannii* isolates produced MBL or OXA-23. All the three antimicrobial combinations, i.e., IPM-CL, RIF-CL, and CAZ-CL, exerted additive/indifferent effects on majority of or all XDR *P. aeruginosa* isolates. And, all the four antimicrobial combinations, i.e., IPM-CL, IPM-AMS, IPM-RIF, and RIF-CL, exerted synergistic or additive/indifferent effects on majority of *A. baumannii* isolates. However, clinical studies should be performed to validate the application of these *in vitro* results in patients because *in vitro* synergy may not be the same as *in vivo* synergy and to determine the exact mechanisms underlying *in vivo* synergy.

Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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